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The microRNA156 and microRNA172 gene regulation cascades at post-germinative stages in *Arabidopsis*

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Abstract

MicroRNAs (miRNAs) are involved in developmental programmes of plants, including seed germination and post-germination. Here, we provide evidence that two different miRNA pathways, miR156 and miR172, interact during the post-germination stages in *Arabidopsis*. Mutant seedlings expressing miR156-resistant *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE13* (*mSPL13*), which has silent mutations in the miR156 complementary sequence, over-accumulated *SPL13* mRNA and exhibited a delay in seedling development. Microarray analysis indicated that *SCHNARCHZAPFEN* (*SNZ*), an *AP2*-like gene targeted by miR172, was down-regulated in these mutants. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) and miRNA gel blot analyses showed that the *MIR172* genes were up-regulated in *mSPL13* mutants. These results suggest that the miRNA regulation cascades (miR156 ⊣ *SPL13* → miR172 ⊣ *SNZ*) play a critical role during the post-germination developmental stages in *Arabidopsis*.

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Introduction

SQUAMOSA PROMOTER-BINDING PROTEIN (SBP) and SBP-LIKE (SPLs) play multiple roles in plant development (Klein *et al.*, 1996; Cardon *et al.*, 1997; Unte *et al.*, 2003; Zhang *et al.*, 2006; see also the accompanying paper Martin *et al.*, 2010). Information concerning the regulatory mechanisms of *SPL* expression is emerging. Through bioinformatic analyses, it is known that 11 out of 17 *Arabidopsis SPLs* contain miR156/miR157 complementary sequences (Rhoades *et al.*, 2002) (miR156 and miR157 are nearly identical, so the term 'miR156' is used hereafter). Targeted cleavage of *SPL3*, *SPL4* and *SPL5* by miR156 has been demonstrated (Chen *et al.*, 2004; Wu and Poethig, 2006). Constitutive expression of *MIR156b* reduces *SPL3* expression, supporting the idea of *SPL* regulation by miRNA (Schwab *et al.*, 2005).

In addition to mRNA cleavage, translational repression of *SPL3* has also been reported (Gandikota *et al.*, 2007). While mRNA cleavage was thought to be the predominant mechanism of miRNA-mediated gene repression in plants, translational repression also appears to be widespread (Brodersen *et al.*, 2008). The *AP2* family genes are regulated by miR172 through translational repression (Aukerman and Sakai, 2003; Chen, 2004), although cleavage of miR172 targets has also been observed (Schwab *et al.*, 2005). Analysis of *SPL3* regulation by miR156 provides another example of the regulation of plant miRNA targets by both mRNA cleavage and translational repression. miR156 complementary sequences have been found in *SPL* orthologues in the moss *Physcomitrella patens*, suggesting an ancient origin of miRNA-dependent regulation of *SPLs* (Arazi *et al.*, 2005; Riese *et al.*, 2007).

While the regulation of *SPLs* by miRNA seems to play a fundamental role in plant growth and development, information on other *SPL* family members targeted by miRNA156 is limited. In this study, we focused on the function of *SPL13*, which is expressed following seed germination. The analysis of the molecular mechanisms of *SPL13* involvement in post-germinative events revealed that miRNA gene regulation cascades function during these stages. Potential interaction between the miR156 and miR172 pathways through *SPL13* function is discussed.

Materials and methods

Generation of miR156-resistant SPL13 *mutants*

The *SPL13* (At5g50570) gene including the 1.3 kb upstream regulatory region was amplified from genomic DNA of *Arabidopsis thaliana* ecotype Col-0 using SPL13 forward (SPL13 F: 5′-ACCTACTCCTGCCAACACAATGTTCTTACA-3′) and reverse (SPL13 R: 5′-ATCCTACAAGATGGCTCATCTCAACAAGGT-3′) primers. The intact gene was used to generate non-mutated *SPL13* transgenic plants. Mutations at the miR156 target site were generated by site overlap extension polymerase chain reaction (PCR) mutagenesis (Ho *et al.*, 1989) using SPL13 mutant forward (SPL13mutF: 5′-CTGATTGTGCTCTCTCACTACTATCTTCCT-3′) and reverse (SPL13mutR: 5′-AGGAAGATAGTAGTGAGAGAGCACAATCAG-3′) primers. PCR products were cloned into pCAMBIA1301. *Arabidopsis thaliana* ecotype Col-0 plants were transformed by floral dip (Clough and Bent, 1998) using *Agrobacterium tumefaciens* carrying pCAMBIA1301 with intact *SPL13* (*SPL13*) or mutant *SPL13* (*mSPL13*) constructs. Wild-type and transgenic plants were grown at 22°C under 12-h light/12-h dark conditions until rosette stages and then flowers were induced by transferring plants to 16-h light/8-h dark conditions.

Differential interference contrast microscopy

Seedlings were cleared with chloral hydrate solution [3.5 ml water, 0.5 g glycerol, 10 g chloral hydrate (Sigma, St. Louis, Missouri, USA)] for 16–18 h. Samples were mounted with Hoyer's solution (7.5 ml water, 1.3 g glycerol, 1.9 g gum arabic and 25 g chloral hydrate) and observed with an Axioskop 2 plus microscope (Zeiss, Jena, Germany). The differential interference contrast (DIC) optics coupled to a Pixera camera Model #PVC 100C (Pixera Corporation, Los Gatos, California, USA) were used to capture images, which were then processed with Pixera Visual Communication Suite software.

mRNA and small RNA extraction

High molecular weight (HMW) RNA for mRNA expression analysis was extracted using a standard phenol–SDS extraction protocol. Briefly, 100 *Arabidopsis* seedlings were homogenized in 2 ml RNA extraction buffer [45.5% (v/v) phenol, 9% (v/v) chloroform, 0.45% (w/v) SDS, 41 mM LiCl, 2 mM EDTA, 5.9 mM β-mercaptoethanol, 82 mM Tris–HCl, pH 8.2] with a mortar and pestle. The extract was centrifuged at 10,000 *g* for 2 min. The supernatant was extracted with one volume of phenol–chloroform–isoamyl alcohol [25:24:1 (v/v/v)] solution and then with one volume of chloroform. LiCl was added to the supernatant (2 M final concentration) and the sample was mixed thoroughly and kept at −20°C overnight. The sample was thawed, mixed and centrifuged at 10,000 *g* for 5 min. The pellet was washed with 1 ml 80% (v/v) ethanol, dried, dissolved in water and used for mRNA expression analysis. To obtain low molecular weight (LMW) RNA for miRNA detection, the supernatant from the 2 M LiCl precipitation step in the total RNA isolation protocol was fractionated by isopropanol (Martin *et al.*, 2005). The pellet from the 35–50% isopropanol fraction was washed with 1 ml 80% (v/v) ethanol, dried, dissolved in water and used for miRNA expression analysis.

Microarray analysis

Three different lots of wild type and three independent transgenic lines of *SPL13* and *mSPL13* were analysed using *Arabidopsis* ATH1 Genome GeneChips (Affymetrix, Santa Clara, California, USA). Seedlings (100) were grown at 22°C under 12-h light/12-h dark conditions and total RNA was extracted from them 3 DAI (days after the start of imbibition). RNA integrity was checked with Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, California, USA). Four micrograms of total RNA from individual pools was used to produce double-stranded cDNAs with Affymetrix One-Cycle Target Labeling Kit, according to the GeneChip Expression Analysis Technical Manual. Biotinylated cRNAs (complementary RNAs) were synthesized from the double-stranded cDNA using T7 RNA polymerase and nucleotide mixture containing biotin-conjugated pseudouridine provided in the IVT Labeling Kit (Affymetrix). cRNA (25 μg) was purified and fragmented prior to hybridization in the Affymetrix GeneChip® Hybridization Oven 640. The arrays were washed in the Affymetrix GeneChip® Fluidics Station 450 and then stained with biotinylated anti-streptavidin (Vector Laboratories, Burlingame, California, USA) at the Center for Genome Research and Biocomputing Core Laboratories at Oregon State University, Corvallis, Oregon, USA. The arrays were

scanned with an Affymetrix GeneChip® Scanner 3000 at 570 nm and signal values were obtained using the statistical algorithms on Affymetrix GeneChip® Operating (GCOS) software. The presence or absence of a reliable hybridization signal for each gene was determined by the detection call on GCOS and imported into GENESPRING GX 7.2 (Agilent Technologies Inc.). The sum of signal values from all probe sets was used for normalization across the different samples. Up- or down-regulated genes were selected when the signal values deviated twofold or more. The microarray data will be available at the Gene Expression Omnibus (GSE10414, http://www.ncbi.nlm.nih.gov/geo).

Quantitative PCR

First-strand cDNA was synthesized from total RNA (1 μg) with QuantiTect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen, Valencia, California, USA). Quantitative reverse transcription (QRT)-PCR with Taq-Man technology (Holland *et al.*, 1991) or SYBR Green I RT-PCR reagents (Qiagen) was performed using the first-strand cDNA as a template on a sequence detector system (ABI PRISM 7000; Applied Biosystems, Foster City, California, USA) as described in a previous report (Yamauchi *et al.*, 2004) with several modifications. Results were normalized using 18S rRNA as the internal control. Duplicate experiments were performed using independent plant materials. Primers used for quantitative PCR were:

SNZ-forward (5′-AGCCTACACAGCCGCAAGA-3′)/reverse (5′-TGGAGTCCCCGGAATCTGA-3′);

MIR172a-forward (5′-TGGCTTCCAAGATCTGGTAATATG-3′)/reverse (5′-ACGAGACAAACCCACAAATTTCTAT-3′);

MIR172b-forward (5′-TGACACGTCAGCCCTTGGA-3′)/reverse (5′-GGGATATGAGGAAAAGTAGATAGGTGAA-3′);

MIR172c-forward (5′-GTCTACATCTATCTCTTTCTAGGTCACTAGCT-3′)/reverse (5′-GCACCATTTTGCTGGAAACA-3′); and

SPL13-qRT-forward (5′-CCTCGTCGTCAGTCCCTCAT-3′)/reverse (5′-TCAACTGCTTCTTGGGACAAAG-3′).

miRNA gel blot

The LMW RNA pellet was dissolved in 2 μl water followed by 4 μl formamide and 2 μl 4 × loading buffer [50% (v/v) glycerol, 0.03% (w/v) bromophenol blue (BPB), 50 mM Tris–HCl, pH 7.7, 5 mM EDTA] and applied to a 17% (w/v) denaturing polyacrylamide gel (85 mm wide, 80 mm long, 1.5 mm thick) containing 7 M urea, 0.5 × Tris–borate–EDTA (TBE) buffer, pH 8.0. The gel was pre-run at 180 V for 30 min. The samples were loaded and the gel was run at 180 V until the BPB line reached the bottom of the gel, stained with ethidium bromide and photographed to visualize the 5S rRNA and tRNA bands. After rinsing the gels with 0.5 × TBE buffer, the separated LMW RNAs were transferred to positively charged Hybond-N + membrane (GE Healthcare Bio-Sciences Corp./Amersham, Piscataway, New Jersey, USA) using a semi-dry transfer unit (Bio-Rad Laboratories, Hercules, California, USA). The transferred RNA was UV cross-linked and the membranes were dried and used for hybridization. miRNA probe synthesis was performed following the instruction manual of the *mir*Vana™ miRNA Probe Construction Kit (Applied Biosystems/Ambion, Austin, Texas, USA). For miRNA probe synthesis, DNA templates were designed based on the miRNA sequence with the addition of part of the T7 promoter sequence (5′-cctgtctc-3′) at the 3′ end of the oligonucleotide. The DNA oligomer and the T7 promoter primer were mixed, heated at 70°C for 5 min and hybridized at room temperature for 5 min. Exo-Klenow DNA polymerase provided with the kit was added to the mix, which was then incubated at 37°C for 30 min to produce a double-stranded DNA template for transcription. Antisense miRNA probes were synthesized at 37°C for 30 min using T7 RNA polymerase (provided with the kit) and a digoxigenin (DIG) RNA-labelling mix (Roche Applied Science, Hague Road, Indianapolis, Indiana, USA). Prehybridization was performed in a hybridization buffer PerfectHyb™ Plus (Sigma-Aldrich) at 42°C for 30 min. Probe (4 μl) was added to 2 ml hybridization solution, heated at 95°C for 5 min and cooled to 42°C. The prehybridization solution was removed and replaced with hybridization solution. Hybridization was allowed to proceed for 16–18 h. The membrane was washed three times at 65°C for 20 min each time with 2 × saline–sodium citrate (SSC), 0.2% (w/v) SDS. Membranes were blocked for 30 min with 5% (w/v) non-fat milk in 0.1 M maleic acid buffer, pH 7.5, containing 0.15 M NaCl, and 0.3% (v/v) Tween 20 (buffer A) and were then incubated with alkaline phosphatase-conjugated anti-DIG antibody for 1 h at 25°C. After washing with buffer A, the membranes were subjected to chemiluminescence detection. The signal was detected on X-ray film after exposure.

Results

SPL13 *represses primordium development post-germination*

Silent mutations created in the *SPL13* sequence that is complementary to the miR156 sequence cause the

deregulation of *mSPL13* from miR156. Transgenic seedlings that overaccumulate the miRNA-resistant mutant *SPL13* (*mSPL13*) exhibited mutant phenotypes visible at post-germinative stages: the development of vegetative leaves was delayed in *mSPL13* mutants (Fig. 1A). In transgenic plants expressing non-mutated *SPL13*, vegetative leaves emerged normally, indicating that the phenotype in *mSPL13* was due specifically to the deregulation of *mSPL13* mRNA from miRNA156. Vegetative leaves became visible only 4–5 DAI even in wild-type seedlings; however, differentiation of leaf primordia seemed to be initiated in very small seedlings right after germination (example of a 2-DAI seedling in Fig. 1A, bottom right). We examined the

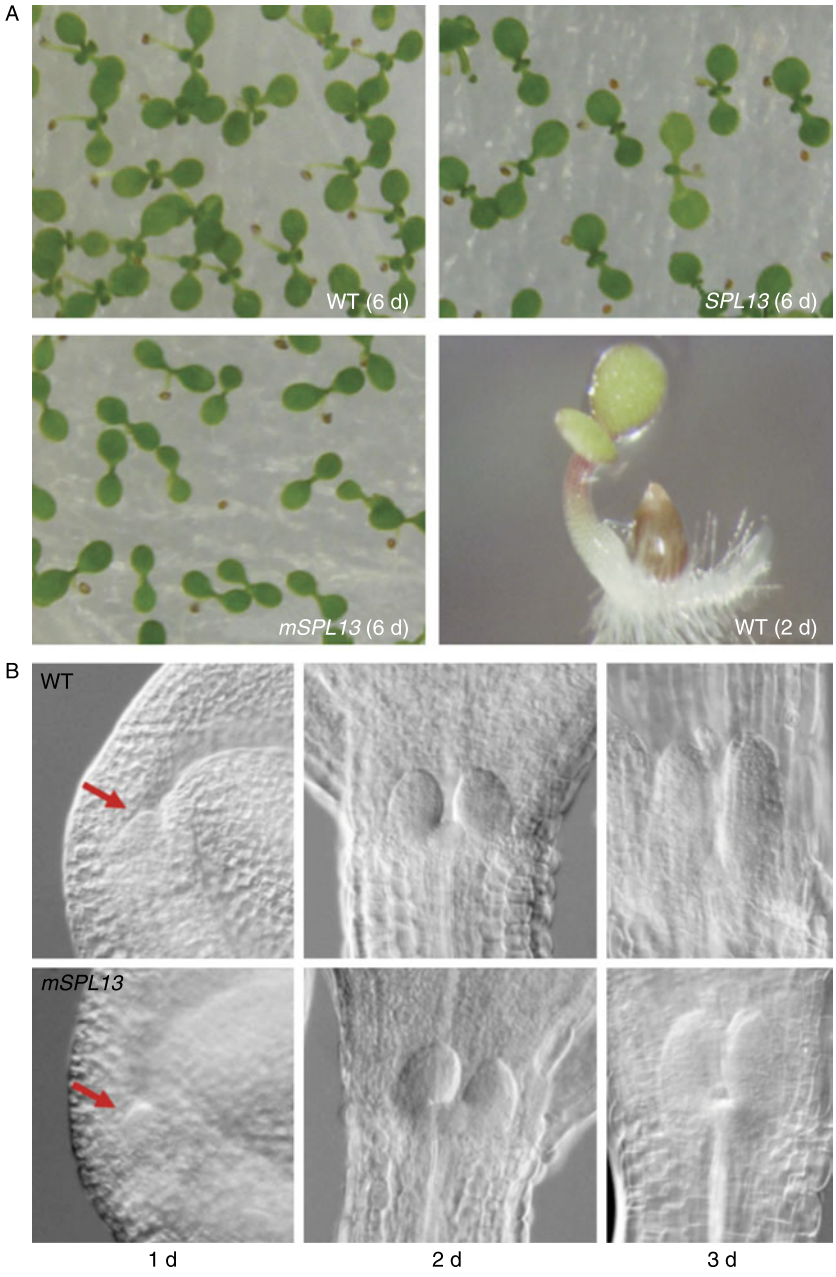

Figure 1. Post-germination phenotypes of miRNA-resistant *mSPL13* mutants. (A) *Arabidopsis* 6-DAI seedlings of wild-type (WT, top left), control transgenic expressing non-mutated *SPL13* (*SPL13*, top right) and mutant plants over-accumulating miR156-resistant *SPL13* (*mSPL13*, bottom left). Note that there was a delay in the emergence of the first pair of vegetative leaves in *mSPL13*. An image of a 2-DAI seedling in which leaf primordia start to differentiate at the shoot apical meristem (see panel B) is also shown (bottom right). (B) Differential interference contrast microscope images of leaf primordia at the shoot apical meristem (SAM) are shown. A slight difference in the size of leaf primordia in WT and *mSPL13* appeared in 3-DAI seedlings. Arrows indicate the position of SAM in a 1-DAI embryo.

Table 1. Genes up-regulated in *mSPL13* mutant seedlings

AGI code	Annotation	Fold change	
		mSPL13/WT	*mSPL13*/*SPL13*
At4g19430	Unknown protein	214.73	300.58
At1g51670	Unknown protein	25.98	11.48
At3g26200	Cytochrome P450 (CYP71B22)	10.58	8.26
At5g50570	Squamosa promoter-binding protein (SPL13)	10.01	2.91
At5g55450	Protease inhibitor/seed storage/lipid transfer protein	6.66	4.83
At1g67865	Unknown protein	6.38	3.44
At1g51460	ABC transporter family protein	6.27	4.32
At1g12010	Similar to ACC oxidase	5.61	4.46
At2g43520	Trypsin inhibitor protein 2	3.57	2.65
At1g52770	Phototropic-responsive NPH3 family protein	3.51	7.66
At1g62500	Protease inhibitor/seed storage/lipid transfer protein	3.44	2.97
At5g55180	Glycosyl hydrolase family 17 protein	2.96	3.17
At5g50600	Short-chain dehydrogenase/reductase (SDR) family	2.92	3.15
At3g30775	Proline dehydrogenase (PRO1)	2.66	2.51

ACC, 1-aminocyclopropane-1-carboxylate.

shoot apical meristems (SAMs) of seedlings at post-germinative stages using a DIC microscope. DIC examination revealed noticeable SAMs in the embryos excised from imbibed seeds. Apparent differentiation of leaf primordia was observed at the SAM around 2 DAI (Fig. 1B). No visible differences were detected between wild-type and *mSPL13* seedlings at this stage. Slight differences in primordia development in the wild type and *mSPL13* were detected 3 DAI (Fig. 1B). These results suggested that the over-accumulation of miR156-resistant *mSPL13* affected the development of the leaf primordia. Based on the information from morphological analysis, seedlings 3 DAI were used for gene expression analysis (see below).

Downstream genes affected by the over-accumulation of mSPL13

The effects of the deregulation of *mSPL13* from miR156 at the molecular level were examined by microarray analysis using RNA extracted from seedlings 3 DAI, when slight morphological differences were visible between wild-type and *mSPL13* seedlings (Fig. 1B). By comparing gene expression in three independent lines of wild-type, *SPL13* and *mSPL13* seedlings, genes up- or down-regulated (twofold or greater) in *mSPL13* mutants were identified. *SPL13* was one of the up-regulated genes which confirmed the deregulation of *mSPL13* from miR156. Other up-regulated genes included plant hormone associated genes, such as 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ethylene biosynthesis) and *NPH3* (auxin response) (Motchoulski and Liscum, 1999), protein inhibitors, a membrane transporter and a dehydrogenase (Table 1). Down-regulated genes also included plant hormone associated genes, such as gibberellin responsive protein GAST1-LIKE and an auxin-induced IAA34 protein. Expansin, a cell wall modifying protein, was also down-regulated in *mSPL13* (Table 2). (Detailed microarray data will be available at the Gene Expression Omnibus, GSE10414, http://www.ncbi.nlm.nih.gov/geo).

Table 2. Genes down-regulated in *mSPL13* mutant seedlings

AGI code	Annotation	Fold change	
		mSPL13/WT	*mSPL13*/*SPL13*
At2g39250	Schnarchzapfen (SNZ)	12.50^{-1}	9.09^{-1}
At1g01390	UDP-glucuronosyl/-glucosyl transferase family	12.50^{-1}	7.69^{-1}
At2g39920	Acid phosphatase class B family protein	5.26^{-1}	5.88^{-1}
At1g74670	Gibberellin-responsive protein (GAST1-LIKE)	4.54^{-1}	4.34^{-1}
At2g01890	Purple acid phosphatase precursor (ATPAP8)	4.34^{-1}	3.57^{-1}
At1g15050	Indoleacetic acid-induced protein 34 (IAA34)	4.00^{-1}	2.70^{-1}
At4g04840	Methionine sulphoxide reductase domain protein	3.70^{-1}	3.44^{-1}
At1g69530	Expansin (ATEXP1)	3.44^{-1}	2.77^{-1}
At1g28600	Carboxylic ester hydrolase	3.22^{-1}	2.85^{-1}

miR156–miR172 gene regulation cascades

One of the genes most significantly down-regulated based on the microarray analysis (nine- to twelvefold decrease) was *SCHNARCHZAPFEN* (*SNZ*), an *AP2-like* gene (Table 2). Quantitative RT-PCR confirmed that *SNZ* was down-regulated specifically in *mSPL13* mutants (Fig. 2A). The *AP2* gene family contains

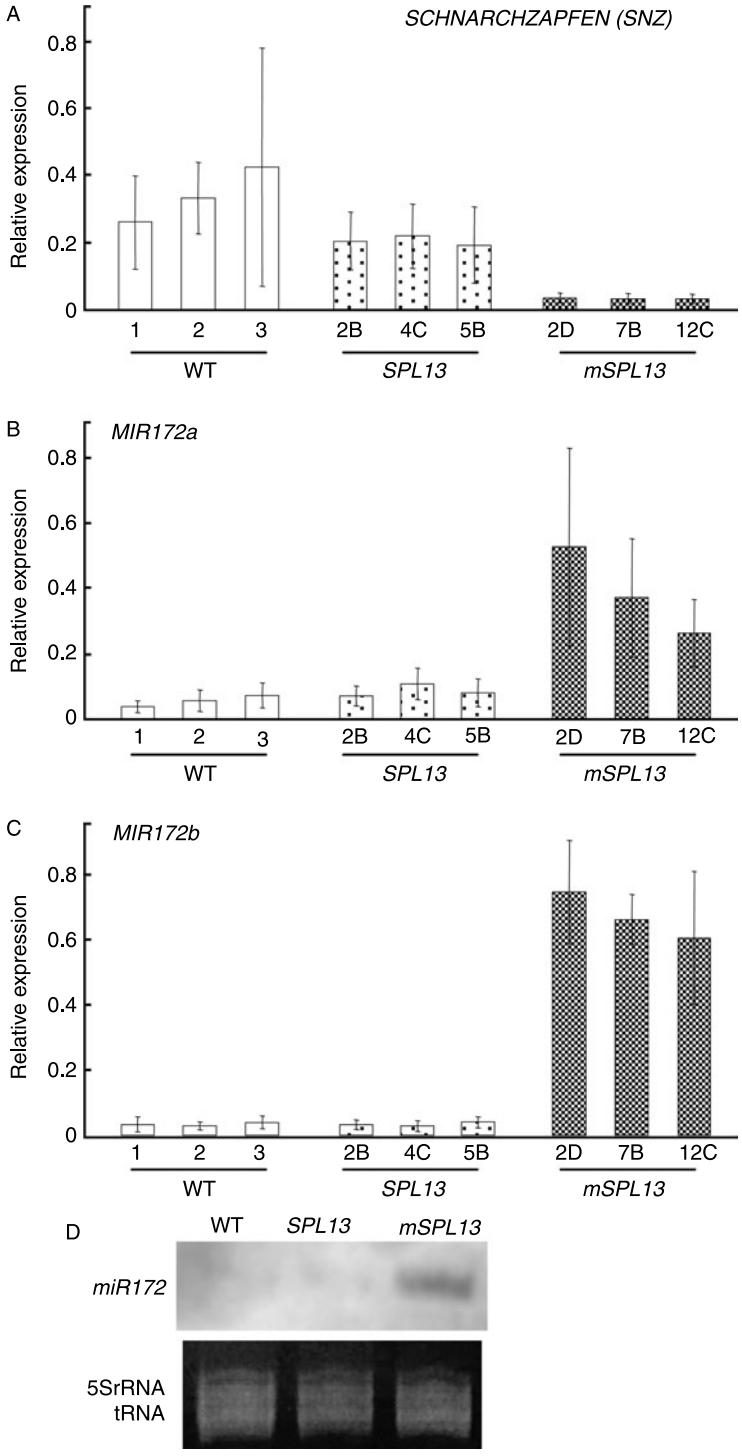

Figure 2. Downregulation of *SCHNARCHZAPFEN* (*SNZ*) and overexpression of *MIR172* genes in *mSPL13* mutants. (A), (B) and (C) Quantitative RT-PCR of *SNZ*, *MIR172a* and *MIR172b*, respectively. Relative gene expression levels in three independent lines of wild type (WT; 1, 2 and 3), control transgenic (*SPL13*; 2B, 4C and 5B) and mutant (*mSPL13*; 2D, 7B and 12C) are shown. Bars indicate SE ($n = 3$). (D) miRNA gel blot for miR172 expression in wild-type (WT), control transgenic (*SPL13*) and mutant (*mSPL13*) seedlings (3 DAI). Results were normalized using 18S rRNA as the internal control.

well-known flower-patterning genes, but *AP2* is also involved in SAM development in *Arabidopsis* seedlings (Wurschum *et al.*, 2006). *SNZ*, another *AP2-like* gene, appears to play a critical role in SAM development.

Interestingly, *SNZ* is a target of miR172 (Schmid *et al.*, 2003) (Fig. 3). Therefore, the microarray data indicated that the deregulation of *SPL13* from miR156 affected a target of miR172. This suggested a potential interaction between miR156 and miR172 pathways. We hypothesized that the down-regulation of *SNZ* in *mSPL13* plants was caused by the up-regulation of miR172 (Fig. 4). We examined the expression of *MIR172*a, *MIR172b* and *MIR172c* predicted gene transcripts (Xie *et al.*, 2005) in three independent lines of wild-type, *SPL13* and *mSPL13* seedlings. While *MIR172c* was barely detectable, *MIR172a* and *MIR172b* were specifically up-regulated in *mSPL13* seedlings (Fig. 2B and C). Based on RNA gel blot of total small RNAs from these lines, mature miR172 over-accumulated in *mSPL13* seedlings (Fig. 2D). These results indicated that over-accumulation of *SPL13* caused over-expression of at least two *MIR172* genes, which resulted in over-accumulation of mature miR172. This also suggested that the down-regulation of *SNZ* in *mSPL13* was caused by this change in miR172 levels.

Discussion

Deregulation of *SPL13* from miR156 caused a delay in the emergence of vegetative leaves at the post-germination stages. The effects of over-accumulation of *SPL13* were partially exerted through the down-regulation of *SNZ*, an *AP2*-like transcription factor (Table 2). The involvement of *AP2*, a floral-patterning gene, in the SAM in *Arabidopsis* seedlings was shown in a previous study (Wurschum *et al.*, 2006). The stem cell niche in the SAM of *Arabidopsis* seedlings is

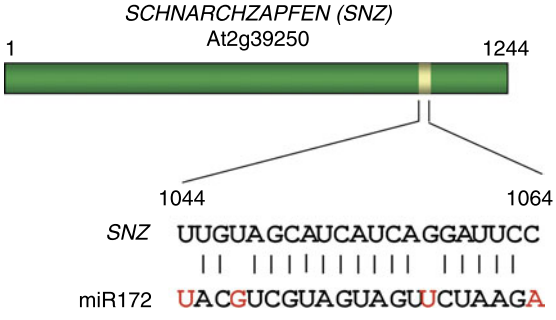

Figure 3. Schematic representation of *SCHNARCHZAPFEN* (*SNZ*) targeted by miR172. The miRNA target sequence in *SNZ* is aligned with the miR172a sequence according to the *Arabidopsis* Small RNA Project (http://asrp.cgrb.oregonstate.edu/). Characters in red indicate mismatch with the *SNZ* sequence.

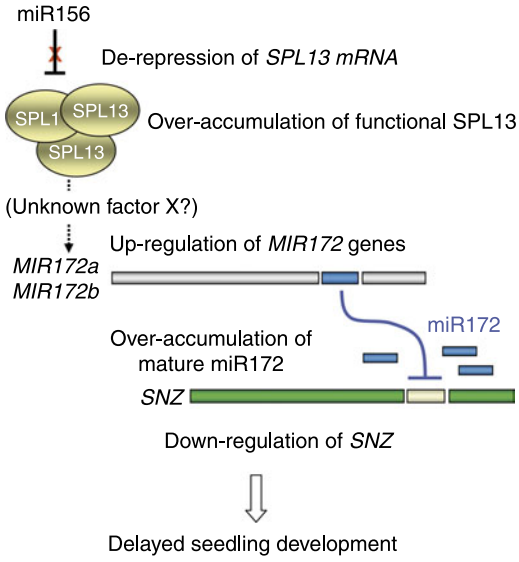

Figure 4. Schematic representation of the miR156–miR172 regulation cascade model in *Arabidopsis* seedlings. Deregulation of m*SPL13* from miR156, due to silent mutations in the miRNA complementary site, causes over-accumulation of functional SPL13, which promotes the expression of *MIR172* genes directly or indirectly through the function of unknown factor X. miR172 then down-regulates *SNZ* which is involved in seedling development. The scheme presents a possible mechanism. A causal connection between *SNZ* down-regulation and the observed phenotype still needs to be verified by further experiments.

maintained primarily by the activity of *WUSCHEL* (*WUS*), a positive regulator, and *CLAVATA3* (*CLV3*), a negative regulator. *AP2* enhances *WUS* activity and reduces *CLV3* activity, both events positively affecting SAM activity (Wurschum *et al.*, 2006). *SNZ* also appears to be a positive regulator of SAM activity. The phenotype of *mSPL13* seedlings where *SNZ* levels were reduced was similar to the phenotype found in *wus* loss-of-function mutant and *CLV3* over-expressers, although the latter mutants have more severe phenotypes than those observed in *mSPL13* seedlings. Therefore, *SNZ* seems to have a role similar to that of *AP2*. It is possible that *SNZ* functions through pathway(s) separate from the *WUS–CLV3* pathway, since microarray data did not indicate a differential expression of these genes between wild-type or control *SPL13* and *mSPL13* seedlings. Microarray data also indicated that multiple genes other than *SNZ* were up- or down-regulated by the over-accumulation of *mSPL13* (Tables 1 and 2). Functional analysis of genes differentially expressed in control and *mSPL13* plants in the present study will provide further information on mechanisms of vegetative leaf development.

In terms of mechanistic analysis, the down-regulation of *SNZ*, a target of miR172, in *mSPL13* mutants suggests the possibility that the miR156

pathway acts upstream of the miR172 pathway in *Arabidopsis*. This possibility was initially predicted by Wu and Poethig (2006) based on complementary patterns of expression of miR156 and miR172 and their function. A recent study demonstrated that *SPL9* mediates the interaction between the miR156 and miR172 pathways (Wu *et al.*, 2009). Our findings provide evidence for the involvement of *SPL13* in the miR156 and miR172 cascades at the post-germinative stages. A similar possibility has been suggested in maize, although the mechanisms are not known. *glossy15* (*gl15*), an AP2 transcription factor loss-of-function mutant in maize (*Zea mays*), exhibits precocious adult cell characteristics in juvenile leaves (Evans *et al.*, 1994; Moose and Sisco, 1994, 1996) suggesting that AP2 family proteins play critical roles in the juvenile-to-adult transition in monocotyledonous species. *Corngrass1* (*Cg1*), a dominant mutant in maize, exhibits prolonged juvenile development. *cg1* encodes two tandem miR156 genes (*zma-MIR156b* and *zma-MIR156c*) that are over-expressed in the meristem and lateral organs in the mutant maize (Chuck *et al.*, 2007). A target of *cg1/zma-MIR156* is *teosinte glume architecture1* (*tga1*), a gene important for the domestication of maize from teosinte (Wang *et al.*, 2005). The expression of *tga1* is reduced in the *Cg1* mutant (Chuck *et al.*, 2007) providing evidence for *tga1* repression by miR156. The tga1 protein contains an SBP domain (data not shown). SPL13 is a tga1 orthologue in *Arabidopsis*. Interestingly, miR172 levels are reduced in *Cg1* mutants (Chuck *et al.*, 2007), supporting the idea that miR172 expression is downstream of the miR156 pathway. These results, together with the results presented here, suggest that SBP-domain-containing transcription factors (SPL13 in *Arabidopsis* or tga1 in maize), which are negatively regulated by miR156, do promote the expression of *MIR172*. miR172 then targets AP2 transcription factors (*SNZ* in *Arabidopsis* or *gl15* in maize) involved in seedling development. Thus, similar miRNA regulation cascades (miR156 ⊣ *SBP-like* → miR172 ⊣ *AP2-like*) appear to be conserved between monocotyledonous and dicotyledonous species.

The mechanisms involved in the induction of *MIR172a* and *MIR172b* by *SPL13* over-accumulation are not known at this time. SPLs are transcription factors that bind conserved DNA motifs in promoter regions of target genes. SPL13 contains an SBP domain that is conserved among other SPLs (Klein *et al.*, 1996). The C-terminal end of this domain contains the bipartite nuclear localization signal (KR… RRRK) which is also found in other SPLs. The conserved DNA motif recognized by *Antirrhinum* SBP and SBP2 and *Arabidopsis* AP1 was first identified as GTCCGTACAA (Klein *et al.*, 1996). Through a more detailed analysis of the binding capacity of the SPL SBP domains, the essential palindromic GTAC core in the motif was identified (Birkenbihl *et al.*, 2005). This motif was found in the 5′ upstream regulatory region (−619/−604) of *MIR172a* (data not shown). However, this motif was not found in the promoter region of *MIR172b* which was also upregulated in *mSPL13* mutants. Therefore, the regulation of the *MIR172* genes by SPL13 may not be direct, although direct control mediated by other motifs is possible.

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